PB-237 691

ISOLATION OF HAYFEVER ANTIGENS FROM SHORT RAGWEED POLLEN

WORTHINGTON BIOCHEMICAL CORPORATION

PREPARED FOR
NATIONAL ENVIRONMENTAL RESEARCH CENTER

26 APRIL 1974

DISTRIBUTED BY:

NTIS
National Technical Information Service
U. S. DEPARTMENT OF COMMERCE

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. R PB 237 691
EPA-650/2-74-044		

4. TITLE AND SUBTITLE	5. REPORT DATE
Isolation of Hayfever Antigens from Short Ragweed Pollen	April 26, 1974
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Robert M. Flora	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Worthington Biochemical Corporation	1AA010
	11. CONTRACT/GRANT NO.
	68-02-0566
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Environmental Protection Agency Chemistry and Physics Laboratory Research Triangle Park, N. C. 27711	Final, Nov. 72 - Mar. 73
	14. SPONSORING AGENCY CODE

15. SUPPLEMENTARY NOTES

16. ABSTRACT

Contract objectives were to isolate antigens from short ragweed pollen for use as standard reference materials in the study of aeroallergens and atmospheric proteins.

Antigen E was prepared in pure form while both of the minor antigens, K and Ra^3, were not processed to purity. Twenty kilogram of pollen were defatted and extracted with water. The antigens were then isolated by salt precipitation and column chromatography. The process was monitored with electrophoresis and immune precipitin tests. The final bulk of antigen E obtained was tested for purity and lyophilized before shipment. Total yield was 5.4g of pure antigen E and approximately 374g of the side fractions containing K and Ra^3. Micro-Ouchterlony tests of antigen E gave a single precipitin line with anti-antigen E serum. Immunoelectrophoresis resulted in a triphasic precipitin band corresponding to the three bands obtained in polyacrylamide gel electrophoresis. UV absorption scans of the antigen E showed a typical protein peak at 230 nm wavelength, and micro-Kjeldahl analysis gave a nitrogen content of 15.4%. Antigens K and Ra^3 were not critically tested. All test results were compatible with published reports and indicated that the antigen E prepared meets a high degree of purity.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Hayfever Ragweed Pollen	Aeroallergens Column Chromatography Isolation of Antigen E	

PRICES SUBJECT TO CHANGE

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release Unlimited	Unclassified	17.
	20. SECURITY CLASS (This page)	22. PRICE
	Unclassified	3.25 - 2.25

EPA Form 2220-1 (9-73)

Reproduced by
NATIONAL TECHNICAL
INFORMATION SERVICE
U S Department of Commerce
Springfield VA 22151

EPA-650/2-74-044

ISOLATION OF HAYFEVER ANTIGENS FROM SHORT RAGWEED POLLEN

by

Robert M. Flora

Worthington Biochemical Corporation
Freehold, New Jersey 07728

Contract No. 68-02-0566
Program Element No. 1AA010

EPA Project Officer: Eva Wittgenstein

Chemistry and Physics Laboratory
National Environmental Research Center
Research Triangle Park, North Carolina 27711

Prepared for

OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

April 1974

This report has been reviewed by the Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Abstract: Short ragweed pollen has been processed to yield antigens associated with hayfever allergy. Antigens K and Ra3 have not been processed to purity but were collected and retained as by-products during the preparation of antigen E. Antigen E has been examined by physical and immunological means and found to meet high standards of purity.

Introduction: A fixed-price contract for $40,000 was established for Worthington Biochemical Corporation to use accepted, published methods for isolating antigens from short ragweed pollen. The basic process uses extraction, salt precipitation, and column chromatography. Process steps are monitored by testing samples with antisera specific for the individual antigens and by polyacrylamide disc gel electrophoresis. Worthington Biochemical Corporation has operated this process several times prior to this run for the Environmental Protection Agency, and was fortunate in encountering no difficulties this time. The process behaved normally and gave yields which fell into the anticipated range.

Materials and Methods

Short ragweed pollen, Lot 18-15-72, consisting of 20 x 1 kg bottles was provided by EPA to the contractor in November of 1972. Information about the handling or drying of the pollen or of its crop year was not provided. The pollen was held in cold storage from the time of arrival until processed.

All chemicals utilized were of reagent grade and suppliers were the following: tris (hydroxymethyl) amino methane (THAM), Fisher Scientific; ethyl ether, sodium chloride, sodium hydroxide and hydrochloric acid, J. T. Baker; ammonium sulfate, J. T. Baker or Mallinckrodt. Column chromatographic media were: Sephadex G-25 and G-100, and DEAE Sephadex A-50, Pharmacia Fine Chemicals; Celite 545, Johns-Manville.

Antisera were from the following sources; anti-antigen E, lot 92008A, and anti-whole ragweed, 1022P, N. I. H. Research Reagents distributed by the National Institute of Allergy and Infectious Diseases; anti-antigen K, 2KA, Dr. T. P. King; anti-antigen Ra3, 2KA, Dr. L. Goodfriend.

Immunodiffusion plates were from Kallestad Laboratories. Immunoelectrophoresis was performed in a thin film agarose medium using films and equipment supplied by Analytical Chemists Incorporated. Disc electrophoresis in polyacrylamide gel and micro-Kjeldahl analysis were conducted in a standard manner by the Worthington Quality Control Department.

Isolation Procedures

Purification of antigen E was based upon methods published by T. P. King and co-workers. (1, 2, 3)

The substitution of DEAE Sephadex A-50 for TEAE cellulose and later DEAE cellulose was a significant difference between the process described by King and the procedure followed by Worthington. One deviation from the technical proposal was required; it was not feasible to carry out recycling gel filtration over Sephadex G-100 because available optical monitoring units could not handle high absorbancies encountered at the scale of this work. An additional, final step not in the original technical proposal was incorporated as an ammonium sulfate gradient/Celite 545 chromatographic procedure more recently described by King.(3)

All chromatographic work was monitored by appropriate immunological techniques, absorbancy at 280nm, and in latter stages by disc gel electrophoresis.

1. Defatting of pollen with ethyl ether

   The 20 kg of pollen was suspended in 60 liters of ethyl ether, slurried manually then mechanically over a period of 30 to 60 minutes followed by transfer to filtration apparatus for solvent removal. The pollen cake was resuspended in 50 liters of ethyl ether and stirred mechanically for 30 minutes, again transferred to filter funnels and the cakes washed extensively with ether until the filtrate was pale yellow. The washed pollen was spread and air-dried until the odor of ether was no longer detectable. The defatting operation was conducted at ambient temperature; all subsequent operations were at 4°C.

2. Extraction of the pollen antigens

   The ether extracted dried pollen was suspended in 100 liters of cold deionized water and stirred mechanically overnight (16-18 hrs). The aqueous extract was collected by filtration, the pH adjusted to 7.0 with 1.0N NH_4OH and brought to cold saturation with ammonium sulfate, 690g/liter. The pollen cake was resuspended in 80 liters of cold deionized water, stirred overnight, filtered, and the second aqueous extract treated as described above. Twice more the pollen cake was extracted with water, 60 liters each time, the extracts neutralized and brought to saturation with ammonium sulfate.

2. Extraction of the pollen antigens (cont'd.)

The aqueous extractible, ammonium sulfate precipitable material was collected by centrifugation, dissolved in water, all four extracts combined, pH adjusted to 7.0, and the solution clarified.

3. Column I; Sephadex G-25

The 9500 ml solution of crude ragweed antigens was desalted and freed of considerable pigment by passage over a 40 x 100 cm bed of Sephadex G-25 equilibrated and eluted with 0.025M Tris-Cl buffer, pH 7.9. All fractions containing appreciable protein and preceding the elution of the ammonium sulfate were pooled.

4. Columns IIa and IIb; DEAE Sephadex A-50

The crude ragweed protein after desalting was applied directly to two in-parallel columns, 21.5 x 100 cm., of DEAE Sephadex A-50 equilibrated with 0.025M Tris-Cl buffer, pH 7.9, followed by washing with the equilibrating buffer until a volume of approximately 60 liters for each column had been collected. Antigens Ra3, Ra5, and BPAP were eluted with the equilibration buffer and these pools were precipitated with ammonium sulfate, dissolved, dialyzed, and lyophilized as the side fractions from columns IIa and IIb. Antigen E and accompanying antigen K were eluted by application of a non-linear gradient derived from 24 liters, each vessel, of equilibration buffer and 0.05M Tris-Cl, 0.25m NaCl, pH 7.9. Fractions containing antigens E and K were combined, precipitated with ammonium sulfate, collected and redissolved in minimal volume and dialyzed against column III buffer.

5. Columns IIIa, IIIb and IIIc; Sephadex G-100

The antigen E and K solution in 0.05m Tris-Cl, 0.4M ammonium sulfate, pH 7.4, (approximately 1200 ml) was applied to a column bed, 21.5 x 100 cm. of Sephadex G-100 packed with the same buffer. Fractions containing antigen E essentially free of antigen K were pooled and held. Fractions containing antigen E contaminated with antigen K were pooled, concentrated, and passed over an identical column, IIIb, of Sephadex G-100. Again antigen E was pooled and held separately from the antigen E and K containing fractions. A third pass over G-100, column IIIc, was performed. Main pools of antigen E from columns IIIa, IIIb and IIIc were

5. Columns IIIa, IIIb and IIIc; Sephadex G-100 (cont'd.)

combined. Leading fractions to the main E pools were combined, precipitated with ammonium sulfate, redissolved, dialyzed, clarified and lyophilized as "leading side fractions", columns IIIabc. Trailing side fractions, not subject to repeat passage over the Sephadex G-100 columns, were treated in a similar manner and were lyophilized as "trailing side fractions" columns IIIabc and should contain the bulk of antigen K.

6. Column IV; DEAE Sephadex A-50

The combined antigen E pool from the previous step was dialyzed against equilibration buffer, 0.025M Tris-Cl, pH 7.9, prior to application to a 10 x 100 cm column bed of DEAE Sephadex A-50. Elution was effected by developing a linear gradient, 10 liters each vessel, of the equilibrating buffer and 0.025M Tris-Cl, 0.4m NaCl, pH 7.9. Fractions containing the substitative portion of the antigen E were combined. Leading and trailing side pools were also made; these were dialyzed and lyophilized as side fractions, column IV.

7. Column V; Celite 545/Ammonium Sulfate Gradient

On the finding that antigen E from column IV had not attained the desired state of purity, an additional chromatographic procedure was employed. A trial run with 5 per cent of the material demonstrated that the ammonium sulfate gradient procedure would further purify antigen E. The bulk of antigen E from column IV in 0.05M Tris-Cl, pH 8.0, was precipitated by 0.7 saturation of ammonium sulfate in the presence of Celite 545. The resulting protein precipitate-Celite slurry was packed into a 5 x 50 cm column over a small plug of Celite. The column was washed with 0.7 saturation of ammonium sulfate following by a gradient, 2 liters each vessel of decreasing ammonium sulfate between the limits of the original conditions down to the simple buffer, 0.05M Tris-Cl, pH 8.0.

The pool of fractions containing the antigen E from column V was extensively dialyzed until free of salt, including the small amount from the trial run and the entire E pool concentrated approximately five-fold in an Amicon ultrafilter device with a 10,000 molecular weight cut-off membrane to a final antigen E concentration of 12 mg/ml.

Antigen K obtained from column V was precipitated by ammonium sulfate, redissolved, dialyzed and lyophilized, and provided as one of the by-product materials.

Antigen E obtained in bulk from the processing described was subjected to final contractor testing as described below. On the determination that it met the required specifications of quality it was prepared for final lyophilization, dispensed into brown 50cc bottles and lyophilized. Fifty-four bottles containing 100 mg. of antigen E were delivered and one bottle, of approximately 50 mg, was retained by the contractor.

Results:

Characterization of Antigen E, Lot 53H382

I Immunological (Micro-Ouchterlony) Analysis

A. Employing specific anti-antigen E serum: Antigen E at concentrations ranging from 1200 to 12 ug/ml vs. anti-antigen E serum (#92008) exhibits a single continuous precipitin line establishing the identity of the material as antigen E. Refer to (Plate I).

B. Employing an anti-whole ragweed serum: Antigen E at concentrations ranging from 1200 to 12 ug/ml vs. anti-whole ragweed serum (#1022P) exhibits again a single continuous precipitin line (Plate II). At the highest level, 1200 ug/ml, another diffuse line is visible. Based upon the level of the sample which shows the contaminant line relative to the concentration of the sample applied it is estimated that the contaminant does not exceed a level of 40 ug/ml or 3.3% of the antigen E. The indication from immunoelectrophoresis is that this contaminant is antigen K. It should be noted that the specification calls for the antigen concentration to be over the range 10 to 200 ug/ml. Where concentrations of the antigen from 12 to 400 ug/ml are used, a contaminant line is not observed.

II Immunoelectrophoresis

Antigen E exhibits a triphasic precipitin line against both anti-antigen E and anti-whole ragweed sera, correlating with the three bands visible in disc polyacrylamide gel electrophoresis. At the level of 12 mg/ml a distinct arc is observed against the anti-whole ragweed serum and a somewhat less distinct line against anti-antigen K serum. At 4 mg/ml the contaminant line is considerably diminished against the anti-whole ragweed serum and almost imperceptible vs. the anti-antigen K serum. Negative results were obtained with anti-antigen Ra3 serum.

III Ultraviolet Spectrum

A scan (Fig. 2) of the antigen E over the wavelength range 400 to 220 nm indicates the preparation possesses an adsorptivity which is typical of protein in general, $^{280}/_{250}$ = 2.40, and characteristic of antigen E in particular. Freedom from significant pigment contamination is indicated by the adsorbancy, $^{280}/_{320}$ = 22.7. Both ratios exceed the contract specifications, which are, $^{280}/_{250} > 2.2$ and $^{280}/_{320} > 15$.

IV Disc Electrophoresis in Polyacrylamide Gel

Antigen E obtained exhibits three bands on disc gel electrophoresis. A densitometric scan (Fig. 3) indicates the bands to be equi-spaced and in the approximate ratio of 15:60:25. Since antigen E may contain as many as four electrophoretically separable components, designated A-D, this finding is compatible with published reports. Preparations conducted over the past 18 months at Worthington Biochemical Corporation have been observed to favor the isolation of more of the A-form and lesser of the D-form of antigen E. Photo 1.

Comparison of lot 53H382 with lot 1KA delivered previously to the EPA reflects a greater degree of antigen E homogeneity as evidenced by freedom from the uppermost band (not in the equi-spaced pattern) and a lesser amount of leading edge smearing. Photo 2.

Admixture of a standard antigen E preparation, XPTA, and lot 53H382 can be reconciled with the following. The standard, primarily forms B and C, with a trace of D band, co-electrophoresised with lot 53H382, some A but mainly B, and C bands, gives the pattern of four visible bands as expected, A, mainly B, C, and a trace of D. Photo 3.

IV Disc Electrophoresis in Polyacrylamide Gel (cont'd.)

The side fraction (Column V; 110mg) containing antigen K is shown compared to a standard antigen K, 3BA. Photo 4.

V Nitrogen Content

Micro-Kjeldahl analysis has determined that the nitrogen content of the antigen E is 15.4%, within the specification range of 15-17%.

Description of Fractions Obtained as By-products, Lot 53H382

Antigens K and Ra3 were not obtained in high states of purity and thus have not been subject to critical analysis. The following fractions obtained as by-products of the Antigen E preparation have been delivered as lyophilized bulks of pooled fractions and are listed with a general description.

1. Side Fractions - Column II; 354 g. Known to contain antigen Ra3 and suspected to contain in addition antigens Ra5 and BPAR. Relatively crude.

2. Leading Side Fractions - Columns IIIa, IIIb and IIIc; 8.6 g. Contains impure antigen E.

3. Trailing Side Fractions - Columns IIIa, IIIb and IIIc; 6.5 g. Contains impure antigen K.

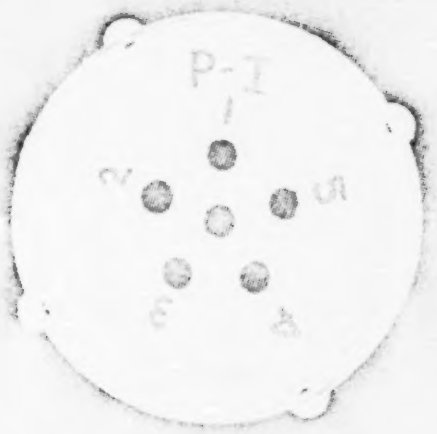
4. Leading Side Fractions - Column IV; 3.0 g. Contains some impure antigen E.

5. Trailing Side Fractions - Column IV; 2.4 g. Contains some antigen E and antigen K.

6. Side Fractions - Column V; 110 mg. Contains relatively pure antigen K.

References

1. King, T. P., Norman, P. S., and Connell, J. T. Biochem. 3, 458 (1964).
2. King, T. P., Norman, P. S., and Lichtenstein, L. M. Biochem. 6, 1992 (1967).
3. King, T. P. Biochem. 11, 367 (1972).



Reproduced from best available copy.

PLATE I

Micro-Ouchterlony analysis employing anti-antigen E serum, 92008A, vs. antigen E, 53H382.

Center well: Anti-antigen E serum

Wells 1-5: 1200, 400, 120, 40, and 12 µg/ml antigen E.

PLATE II

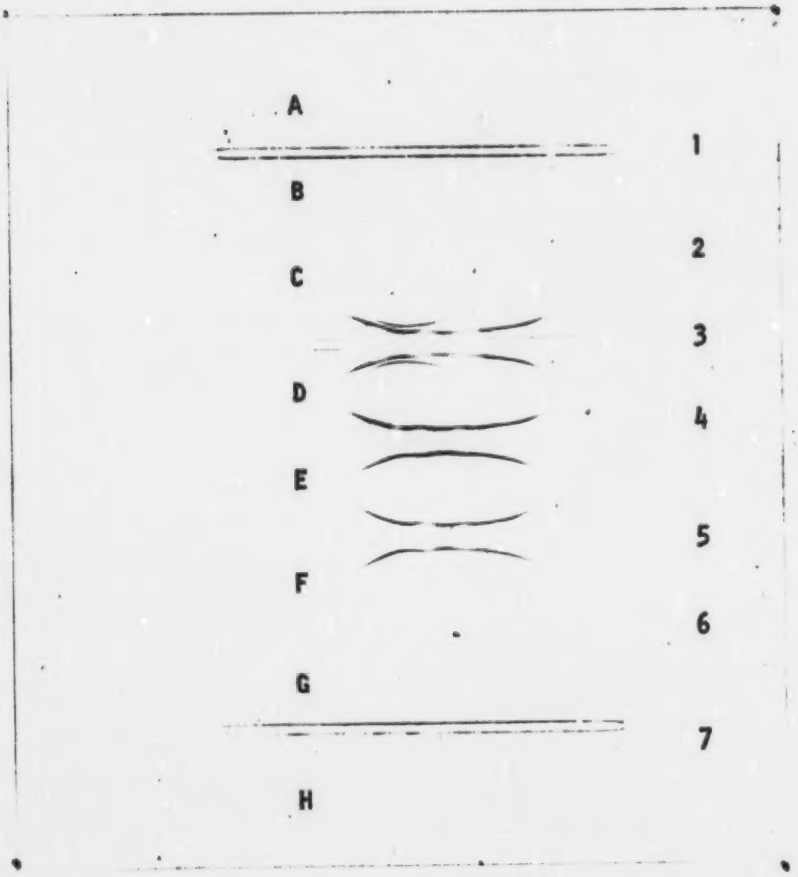
Micro-Ouchterlony analysis employing anti-whole ragweed serum, 1022P, vs. antigen E, 53H382.

Center well: Anti-whole ragweed serum

Wells 1-5: 1200, 400, 120, 40, and 12 µg/ml antigen E

FIGURE 1

THIN FILM AGAROSE IMMUNOELECTROPHORESIS



Wells A-D: Antigen E, 53H382, 12 mg/ml; 2 microliters in each well.

Wells E-H: Antigen E, 53H382, 4 mg/ml; 2 microliters in each well.

Trough 1 and 7: Anti-antigen Ra3 serum, lot 2KA

Trough 2 and 6: Anti-antigen K serum, lot 2KA

Trough 3 and 5: Anti-whole ragweed serum, #1022P

Trough 4 : Anti-antigen E serum, #92008A

(Approximately 40 microliters serum applied to trough)

FIGURE 2

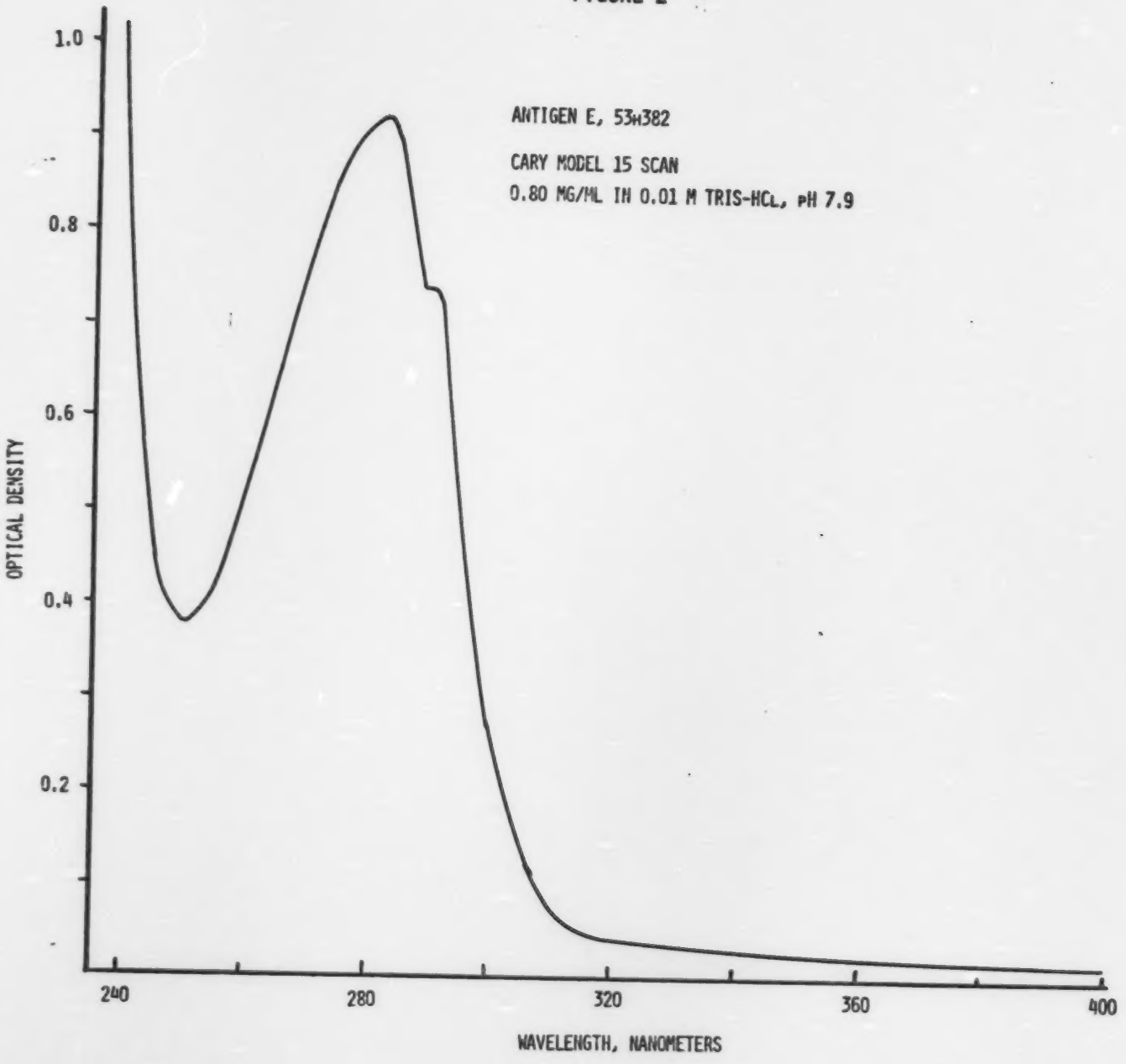
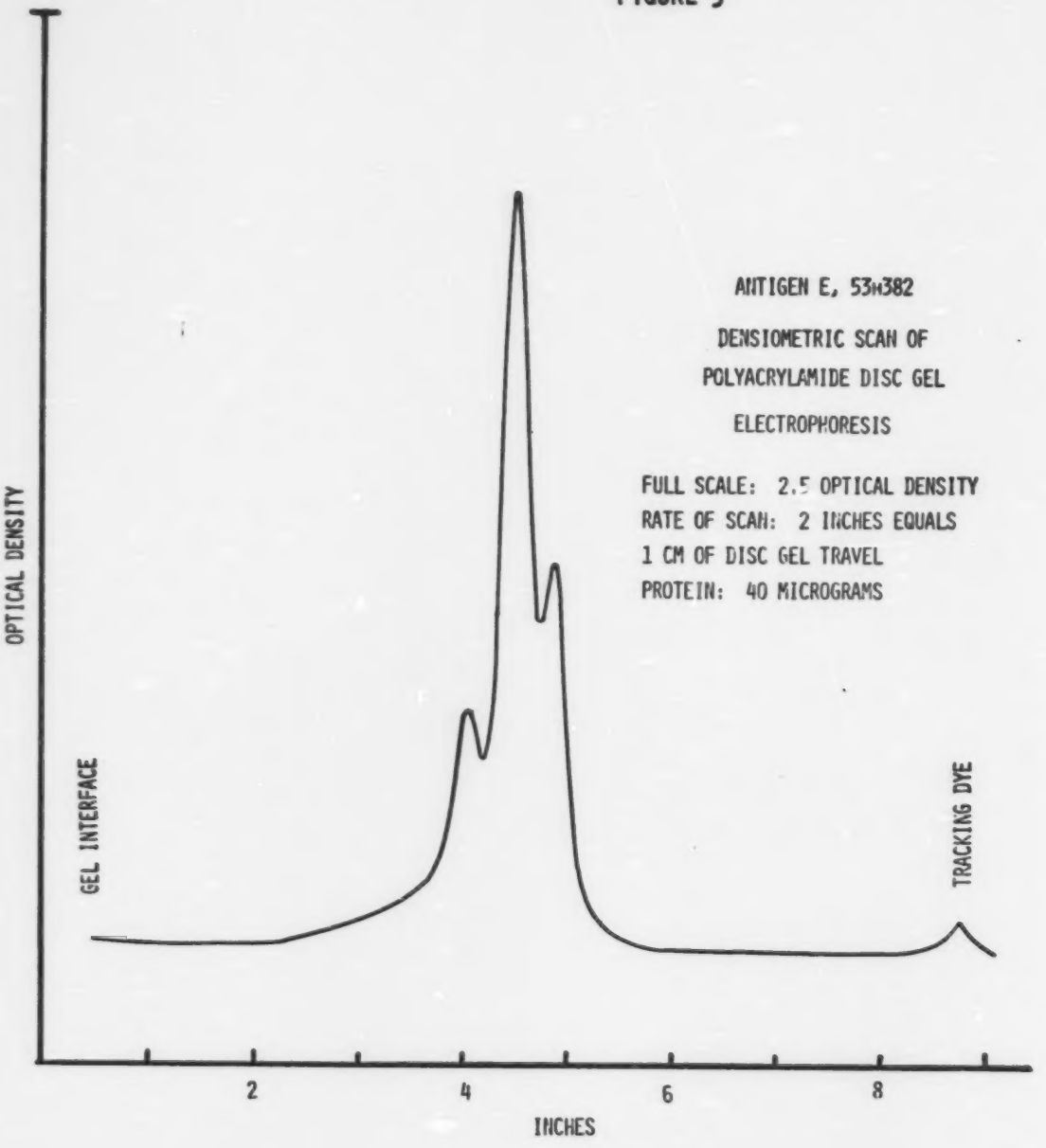

ANTIGEN E, 53H382
CARY MODEL 15 SCAN
0.80 MG/ML IN 0.01 M TRIS-HCL, pH 7.9
OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
240
280
320
360
400
WAVELENGTH, NANOMETERS

FIGURE 3


ANTIGEN E, 53H382
DENSIOMETRIC SCAN OF
POLYACRYLAMIDE DISC GEL
ELECTROPHORESIS
FULL SCALE: 2.5 OPTICAL DENSITY
RATE OF SCAN: 2 INCHES EQUALS
1 CM OF DISC GEL TRAVEL
PROTEIN: 40 MICROGRAMS
OPTICAL DENSITY
GEL INTERFACE
TRACKING DYE
2
4
6
8
INCHES

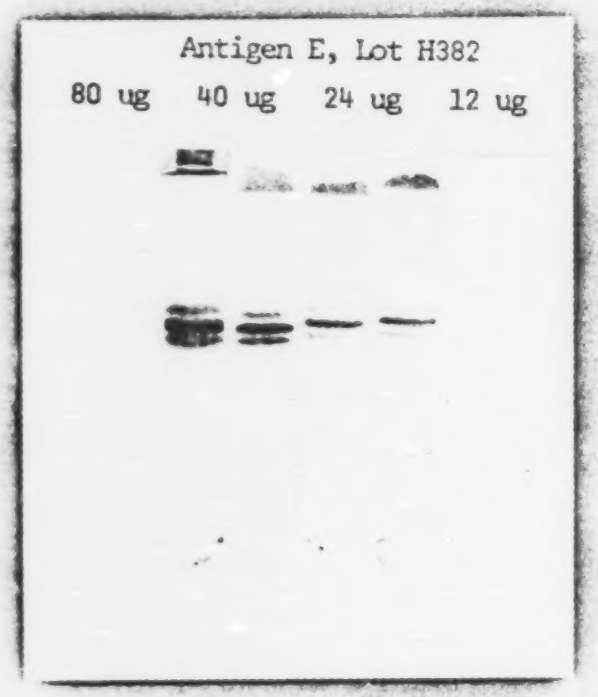


PHOTO 1

Antigen E, 53H382, polyacrylamide disc gel electrophoresis, pH 8.6. Micrograms antigen E applied, left to right, 80, 40, 24 and 12.

PHOTO 2

Antigen E, lot IKA and lot 53H382

Reproduced from best available copy.

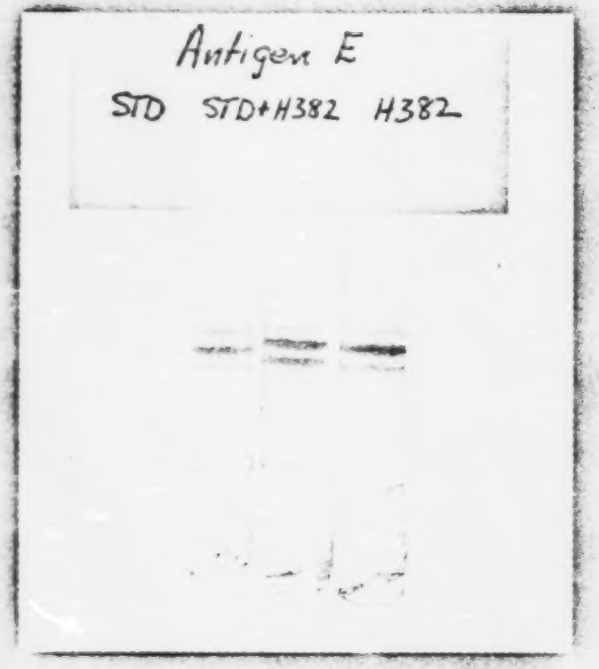


PHOTO 3

Antigen E, left, standard, lot 01A, right, lot 53H382, and center, mixture of 01A and 53H382.

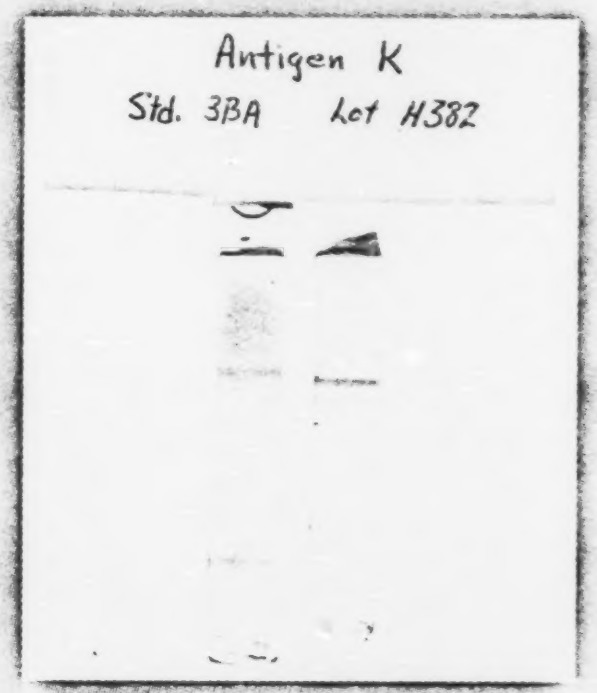


PHOTO 4

Antigen K, 53H382, a by-product of Column V, compared with an antigen K standard.

Reproduced from best available copy.